Effects of dietary supplementation with α -linolenic acid on the phospholipid fatty acid composition and quality of spermatozoa in cockerel from 24 to 72 weeks of age

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The aim of this study was to investigate the effects of supplementing the diet of the male chicken with α -linolenic acid (18:3n-3) on the phospholipid fatty acid composition, motility and fertilizing ability of chicken spermatozoa. The birds in the control group received a diet supplemented with soybean oil rich in linoleic acid (18:2n-6) whereas those in the test group were supplemented with linseed oil rich in α -linolenic acid. A number of age-related changes in the lipid parameters of the spermatozoa were observed in control birds. Between 24 and 72 weeks of age the amount of total lipid in the spermatozoa of control birds increased by approximately 2.4 times and the proportions of cholesterol and free fatty acid also increased significantly, whereas the proportions of phospholipid and triacylglycerol decreased. In addition, the proportion of phosphatidylcholine in the total phospholipid increased, whereas the proportion of phosphatidylserine decreased during the same period. The proportion of docosatetraenoic acid (22:4n-6) in the phospholipid decreased significantly between 24 and 72 weeks of age. The concentration of spermatozoa in the semen of control birds increased to a maximum at week 39 and had decreased significantly by week 72. Supplementation with α -linolenic acid had little or no effect on the proportion of docosahexaenoic acid (22:6n-3) in the phospholipid profile of the spermatozoa. However, supplementation with α -linolenic acid did produce a significant but small increase in the proportion of docosapentaenoic acid (22:5n-3) recorded at 39 and 54 weeks. Thus, this study shows that the fatty acid composition of the sperm phospholipid demonstrates a marked resistance to dietary manipulation. Supplementation with α -linolenic acid significantly enhanced semen fertility at week 39. The results suggest that the small increase in the proportion of n-3 fatty acids in the sperm phospholipids induced by enriching the diet with α -linolenic acid is associated with a significant improvement in semen quality at 39 weeks of age.

Introduction

The phospholipids of mammalian spermatozoa display a highly characteristic fatty acid composition, the most striking feature of which is the presence of extremely high proportions of docosahexaenoic acid (22:6n-3) (Neill and Masters, 1972; Poulos and White, 1973; Poulos *et al.*, 1973; Scott, 1973; Jain and Anand, 1976; Salem *et al.*, 1986; Lin *et al.*, 1993). This long-chain, highly polyunsaturated fatty acid is also present at very high concentrations in the phospholipids of the retina and brain (Salem *et al.*, 1986; Neuringer *et al.*, 1988). In contrast to mammals, spermatozoa from avian species that have so far

been studied display extremely low concentrations of docosahexaenoic acid in their phospholipids. Instead, the major fatty acyl components of avian spermatozoa are arachidonic (20:4n-6) and docosatetraenoic (22:4n-6) acids (Darin-Bennett *et al.*, 1974; Howarth *et al.*, 1977; Ravie and Lake, 1985; Kelso *et al.*, 1996). Thus, avian spermatozoa are characterized by high amounts of C_{20–22} polyunsaturates of the n-6 series, whereas long-chain fatty acids of the n-3 series predominate in mammalian spermatozoa. However, it should be noted that the compositional data for avian spermatozoa are almost wholly confined to domestic poultry species (the chicken and the turkey) and that little information is available for birds living in the wild. Since commercial poultry production currently relies on compounded feeds that generally contain a vast excess of n-6 fatty acids (mainly linoleic acid (18:2n-6)) over n-3 fatty

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acids (Noble, 1986), it is conceivable that the reported fatty acid composition of avian spermatozoa is a dietary-induced displacement from the 'natural' pattern rather than a true phylogenetic difference from mammals.

It is well known that animals deprived of a source of n-3 fatty acids at critical stages of embryonic or neonatal development display greatly reduced concentrations of 22:6n-3 in brain and retina phospholipids and, by way of compensation, accumulate high proportions of long-chain n-6 fatty acids such as 22:4n-6 and 22:5n-6 (Salem *et al.*, 1986; Neuringer *et al.*, 1988; Anderson *et al.*, 1989). Thus, the characteristic fatty acid profiles of poultry spermatozoa are typical of those associated with n-3 deficiency. There is considerable evidence that 22:6n-3 performs an essential role in the functional maturation of tissues such as the brain and retina and that its replacement by 22:4n-6 and 22:5n-6 during n-3 deficiency is unable to prevent a range of functional impairments (Neuringer *et al.*, 1988; Sanders *et al.*, 1988; Innis, 1991). Similarly, it appears that the 22:6n-3 present in mammalian spermatozoa also performs an essential function in promoting optimal fertility, as marked reductions in the amount of this fatty acid in spermatozoa are associated with impaired sperm number, motility and fertilizing ability (Nissan *et al.*, 1981; Nissan and Kreysel, 1983). If the typical fatty acid composition currently observed for the spermatozoa of domestic poultry is simply an aberration due to a dietary imbalance of n-3 versus n-6 fatty acids, the fertility of poultry might be amenable to improvement via appropriate dietary manipulation.

Materials and Methods

Dietary supplementation of cockerels and semen collection

Male chickens (Cobb broiler-breeder strain) were purchased at 21 days of age from a commercial poultry supplier. The birds were housed in single cages in a controlled environment with a photoperiod of 13 h light:11 h dark. They were each fed 130 g feed day⁻¹ containing 12.5% (w/w) crude protein and 11.5 MJ metabolizable energy kg⁻¹. From 23 to 72 weeks of age, 15 birds received the control diet, which was supplemented with soybean oil rich in linoleic acid (18:2n-6) (6% (w/w) of feed); another 15 birds received the diet supplemented with linseed oil rich in α -linolenic acid (18:3n-3) (6% (w/w) of feed). The total fat content of both diets was 9% (w/w) of feed. The fatty acid compositions of the two diets are shown (Table 1). Both diets contained vitamin E at a concentration of 40 mg kg⁻¹ feed. Fresh batches of the respective feeds were produced weekly and regular analysis revealed that no significant changes in the fatty acid compositions occurred throughout the duration of the experiment. There were no significant effects of dietary supplementation with 18:3n-3 on the growth and body masses of the birds, for example, at 72 weeks of age the mean body masses (g) of the control and supplemented birds were 4555 $\pm$ 429 g and 4654 $\pm$ 384 g (n = 15), respectively.

The birds were trained for semen collection (Lake and Stewart, 1978) from 21 weeks of age. Two semen samples were collected per week after this age, apart from the weeks chosen for analysis (24, 39, 54 and 72 weeks of age) when three samples were taken.

Table 1. Fatty acid compositions of the control diet supplemented with soybean oil rich in linoleic acid (18:2n-6) and of the diet supplemented with linseed oil rich in α -linolenic acid (18:3n-3) in male chickens

Fatty acids (% w/w)	Supplementation	
	Soybean oil	Linseed oil
16:0	12.1 $\pm$ 0.3	8.7 $\pm$ 0.2
18:0	3.5 $\pm$ 0.1	3.3 $\pm$ 0.1
18:1n-9	23.4 $\pm$ 0.1	20.7 $\pm$ 0.2
18:2n-6	52.9 $\pm$ 1.9	28.8 $\pm$ 0.3
18:3n-3	6.2 $\pm$ 0.1	36.8 $\pm$ 1.5
Ratio of 18:2n-6 to 18:3n-3	8.5 $\pm$ 0.2	0.78 $\pm$ 0.1

Values are means $\pm$ SD of measurements taken from four samples of feed.

Only the major fatty acids (> 0.5%, w/w) are shown.

No C₂₀₋₂₂ fatty acids were detected (limits of detection were approximately 0.1%, w/w).

Evaluation of semen quality

Within 20 min of collection, the semen volume was measured using a graduated tube, and sperm concentration and motility were determined using a Cellsoft Computer-Assisted Digital Imaging System (CellSoft[®] Version 3, Cryoresources Ltd, New York). Motility was expressed as the percentage of the total number of spermatozoa that were motile. The fertilizing ability of the semen was assessed by artificially inseminating laying hens. The hens (ISA-Warren strain) were purchased at 21 weeks of age, housed in single cages in a controlled environment of 16 h light:8 h dark and fed freely with a standard layer diet (11.5 MJ metabolizable energy kg⁻¹ feed and 18% (w/w) crude protein). At each time of insemination, the males and females were the same age. Semen samples were diluted with two volumes of 0.9% (w/w) NaCl and a single dose of spermatozoa (approximately 7×10^7) was inseminated within 20 min of semen collection. Semen from groups of three males was pooled to provide five replicate pooled samples per dietary treatment. Each pooled sample of semen was used to inseminate seven hens, and eggs were collected over the next seven days. At each of the four ages studied, hens were selected at random to receive semen from either the control or the supplemented males. The eggs were incubated at 37.5°C and 60% relative humidity and were turned automatically for 7 days. The eggs were then opened and the numbers of those containing live, dead or no embryos were recorded. Only those containing no embryos were classed as infertile.

Lipid extraction and analysis

Semen samples from groups of three cockerels were pooled to obtain sufficient material for lipid analysis, five replicate pooled samples were used for each of the dietary treatments. Semen samples were diluted with an equal volume of 0.85% (w/v) NaCl and centrifuged at 700 g for 20 min at 4°C. The upper diluted seminal plasma layer was discarded; the wash procedure was repeated with 1 ml 0.85% (w/v) NaCl solution

Table 2. Characteristics of semen from cockerels on a control diet rich in linoleic acid (18:2n-6) and from birds on a diet supplemented with α -linolenic acid (18:3n-3) at 24, 39, 54 and 72 weeks of age

Semen characteristic	24 weeks		39 weeks		54 weeks		72 weeks	
	Control	n-3 diet	Control	n-3 diet	Control	n-3 diet	Control	n-3 diet
Semen volume (ml) ^a	1.9 ± 0.1 ^d	1.8 ± 0.1 ^d	1.4 ± 0.1 ^e	1.4 ± 0.1 ^e	1.7 ± 0.2 ^f	1.1 ± 0.1 ^f	1.0 ± 0.2 ^g	1.1 ± 0.2 ^f
Sperm concentration (10 ⁹ cells ml ⁻¹)	4.7 ± 0.4 ^d	5.0 ± 0.4 ^d	7.7 ± 0.4 ^e	7.1 ± 0.5 ^e	6.3 ± 0.7 ^d	6.7 ± 0.5 ^e	1.9 ± 0.7 ^f	2.0 ± 0.6 ^f
Motility (%) ^b	56.4 ± 4.4 ^d	56.5 ± 4.4 ^d	54.2 ± 4.4 ^d	57.9 ± 5.4 ^d	40.2 ± 7.8 ^d	48.3 ± 5.4 ^d	63.1 ± 7.8 ^d	41.4 ± 6.3 ^{dh}
Fertility (%) ^c	68.3 ± 6.8 ^d (195)	66.7 ± 10.4 ^d (189)	82.8 ± 6.8 ^e (194)	96.6 ± 10.4 ^{eh} (151)	74.4 ± 6.8 ^d (151)	80.1 ± 8.3 ^d (157)	65.4 ± 10.1 ^d (99)	69.5 ± 8.6 ^d (106)

^aSemen volume refers to the total volume of the pooled semen from three birds.

^bMotility is the percentage of the total number of spermatozoa that were motile.

^cFertility is the percentage of the number of eggs (given in parentheses) containing embryos that were laid by hens artificially inseminated with the spermatozoa.

^{d,e,f,g}For comparisons between ages for birds on the same diet, values with different superscripts are significantly different ($P < 0.05$).

^hDenotes significant difference ($P < 0.05$) between diets for birds of the same age.

Value are means ± SD.

and the final cell pellet was resuspended in 2 ml 0.85% (w/v) NaCl solution.

Total lipids were extracted from the washed cell preparations after homogenization in a suitable excess of chloroform:methanol (2:1, v:v) (Christie, 1982). The lipids were fractionated into their major classes (phospholipid, triacylglycerol, cholesterol, cholesteryl ester and free fatty acid) by thin layer chromatography on silica gel G using a solvent system of hexane:diethyl ether:formic acid (80:20:1 v:v:v). After spraying the gel with 2,7-dichlorofluorescein (0.1% (w/v) in methanol), the bands were visualized under the UV light and the separated bands were then scraped from the plates. Phospholipid was eluted from the silica by washing it three times with 2 ml methanol; the other lipid classes were similarly eluted using diethyl ether. The isolated lipid classes (with the exception of cholesterol) were subjected to *trans*methylation by refluxing for 30 min with methanol:toluene:sulfuric acid (20:10:1, v:v:v) in the presence of a pentadecanoic acid (15:0) standard (Christie *et al.*, 1970). The resultant fatty acid methyl esters were analysed by injecting 2 μ l, via a CP9010 auto sampler (Chrompack Ltd, London), on to a Carbowax capillary column 30 m $\times$ 0.25 mm wide, with a film thickness of 0.25 μ m (Alltech UK Ltd, Camforth) fitted within a Chrompack CP9001 gas chromatograph (Chrompack Ltd). The fatty acid compositions (percentage (w/w) of total fatty acids) were derived by integrating the peaks using an EZ Chrom data handling system (Speck Analytical Ltd, Alloa). The absolute amounts of the acyl-containing lipid classes were also calculated using this integration method (Christie *et al.*, 1970). Cholesterol was determined by an enzymic-colorimetric method using a kit system (Boehringer Ltd, Lewes).

The individual phospholipid classes (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, cardiolipin and sphingomyelin) were separated by high performance thin layer chromatography using a solvent system of methyl acetate:isopropanol:chloroform:methanol:0.25% (w/v in H₂O) KCl (25:25:25:19:9, v:v:v:v), as described by Olsen and Henderson (1989). After charring, the phospholipids were quantified by densitometry using a Shimadzu CS-9001, dual-wavelength, flying spot scanner (V. A. Howe Ltd, Banbury).

Statistical analysis

Statistical comparisons were based on 15 replicate semen samples (each sample derived from an individual bird) from each dietary group, with respect to data on semen volume, cell concentration and motility. Statistical analyses of the fertility values and of the lipid composition parameters were based on five replicate pooled semen samples from each dietary group, with each pooled sample obtained from three individual birds. The data was assessed by analysis of variance (ANOVA) using the General Linear Model method. All percentage data were arcsin transformed prior to ANOVA (Snedecor and Cochran, 1967).

Results

For the cockerels on the control diet, the concentration of spermatozoa in the semen increased significantly between 24 and 39 weeks of age and then decreased markedly between 54 and 72 weeks (Table 2). Dietary supplementation with 18:3n-3 had no effect on semen volume or sperm concentration at any stage but produced a significant decrease in motility at 72 weeks. The fertility of the semen obtained from the control birds was maximal at 39 weeks but had decreased considerably by 72 weeks. The n-3 supplementation resulted in a significant increase in fertility at 39 weeks (Table 2).

The concentration of total lipid in the spermatozoa cells increased with age for both the control and the birds supplemented with 18:3n-3, with a significant difference between the dietary groups detected only at 72 weeks (Table 3). The proportion of phospholipid was maximal at 39 weeks but decreased considerably thereafter, with no differences between the dietary groups. The proportion of cholesterol was significantly higher at the later stages and at 39 and 72 weeks was increased by supplementing the diet with 18:3n-3. The concentration of free fatty acids in the spermatozoa was significantly greater at 72 weeks than at 24 weeks for the birds on the control diet. The proportion of cholesteryl ester was at a minimum at 39 weeks for both dietary groups and was also

Table 3. The proportions of the major lipid classes in spermatozoa from cockerels on a control diet rich in linoleic acid (18:2n-6) and from birds on a diet supplemented with α -linolenic acid (18:3n-3) at 24, 39, 54 and 72 weeks of age

Lipid	24 weeks		39 weeks		54 weeks		72 weeks	
	Control	n-3 diet	Control	n-3 diet	Control	n-3 diet	Control	n-3 diet
Total lipid (μg per 10^9 cells)	290 $\pm$ 51 ^{b,c}	261 $\pm$ 51 ^b	275 $\pm$ 51 ^b	316 $\pm$ 58 ^b	364 $\pm$ 56 ^c	463 $\pm$ 58 ^c	694 $\pm$ 66 ^d	556 $\pm$ 66 ^{c,e}
Lipid class (%) ^a								
Phospholipid	62.8 $\pm$ 3.8 ^b	68.3 $\pm$ 6.6 ^b	69.2 $\pm$ 2.9 ^c	68.5 $\pm$ 3.3 ^b	57.3 $\pm$ 4.6 ^{b,d}	62.1 $\pm$ 3.8 ^b	51.0 $\pm$ 3.8 ^d	50.8 $\pm$ 3.8 ^c
Cholesterol	12.6 $\pm$ 2.3 ^b	10.0 $\pm$ 3.9 ^b	12.9 $\pm$ 1.7 ^b	17.8 $\pm$ 2.0 ^{c,e}	22.0 $\pm$ 2.8 ^c	23.6 $\pm$ 2.3 ^d	16.4 $\pm$ 2.2 ^d	24.3 $\pm$ 2.3 ^{d,e}
Cholesterol ester	11.5 $\pm$ 3.1 ^b	11.0 $\pm$ 5.3 ^b	7.2 $\pm$ 3.4 ^b	5.2 $\pm$ 2.7 ^b	9.0 $\pm$ 3.8 ^b	6.2 $\pm$ 3.1 ^b	18.9 $\pm$ 3.1 ^c	12.0 $\pm$ 5.1 ^{b,e}
Free fatty acid	5.9 $\pm$ 2.1 ^b	7.4 $\pm$ 3.6 ^{b,c}	6.9 $\pm$ 1.6 ^b	5.3 $\pm$ 1.8 ^c	9.1 $\pm$ 2.6 ^{b,c}	5.6 $\pm$ 2.1 ^c	11.4 $\pm$ 2.1 ^c	10.0 $\pm$ 2.1 ^b
Triacylglycerol	7.3 $\pm$ 1.4 ^b	3.3 $\pm$ 2.4 ^{b,e}	3.8 $\pm$ 1.1 ^c	3.2 $\pm$ 1.2 ^b	2.5 $\pm$ 1.7 ^c	2.6 $\pm$ 1.4 ^b	2.3 $\pm$ 1.4 ^c	2.9 $\pm$ 1.4 ^b

^aThe lipid class values refer to the percentage (w/w) of the total lipid (values are means $\pm$ SD).

^{b,c,d}For comparisons between ages for birds on the same diet, values without a superscript in common are significantly different ($P < 0.05$).

^eDenotes significant difference ($P < 0.05$) between diets for birds of the same age.

Table 4. The proportions of the major phospholipid classes in spermatozoa from cockerels on a control diet rich in linoleic acid (18:2n-6) and from birds on a diet supplemented with α -linolenic acid (18:3n-3) at 24, 39, 54 and 72 weeks of age

Phospholipid ^a	24 weeks		39 weeks		54 weeks		72 weeks	
	Control	n-3 diet	Control	n-3 diet	Control	n-3 diet	Control	n-3 diet
Phosphatidylcholine	32.3 $\pm$ 1.1 ^b	33.4 $\pm$ 1.1 ^b	25.4 $\pm$ 1.1 ^c	27.5 $\pm$ 1.3 ^c	34.8 $\pm$ 1.1 ^d	33.2 $\pm$ 1.3 ^b	42.3 $\pm$ 1.5 ^e	40.0 $\pm$ 1.3 ^d
Phosphatidylethanolamine	33.1 $\pm$ 1.4 ^b	31.7 $\pm$ 1.4 ^b	33.8 $\pm$ 1.4 ^b	32.9 $\pm$ 1.7 ^b	31.1 $\pm$ 1.4 ^b	32.9 $\pm$ 1.7 ^b	27.4 $\pm$ 2.0 ^c	30.1 $\pm$ 1.7 ^{b,f}
Phosphatidylserine	19.2 $\pm$ 1.3 ^b	18.9 $\pm$ 1.3 ^b	24.4 $\pm$ 1.3 ^c	21.8 $\pm$ 1.5 ^c	20.9 $\pm$ 1.3 ^b	21.1 $\pm$ 1.5 ^b	11.8 $\pm$ 1.7 ^d	14.1 $\pm$ 5.4 ^d
Sphingomyelin	10.5 $\pm$ 1.4 ^b	11.9 $\pm$ 1.4 ^b	11.5 $\pm$ 1.4 ^c	12.1 $\pm$ 1.7 ^b	8.4 $\pm$ 1.4 ^c	8.6 $\pm$ 1.7 ^c	14.1 $\pm$ 1.9 ^d	11.3 $\pm$ 1.7 ^{b,f}
Cardiolipin	4.9 $\pm$ 0.4 ^b	4.1 $\pm$ 0.4 ^b	4.9 $\pm$ 0.4 ^b	5.7 $\pm$ 0.5 ^c	4.9 $\pm$ 0.4 ^b	4.2 $\pm$ 0.5 ^b	4.5 $\pm$ 0.6 ^b	4.5 $\pm$ 0.5 ^b

^aThe values refer to the percentage (w/w) of the total phospholipid (values are means $\pm$ SD).

^{b,c,d,e}For comparisons between ages for birds on the same diet, values without a superscript in common are significantly different ($P < 0.05$).

^fDenotes significant difference ($P < 0.05$) between diets for birds of the same age.

significantly reduced at 72 weeks by the n-3 supplementation (Table 3). The proportion of phosphatidylcholine decreased to a minimum value at 39 weeks and increased thereafter, whereas the proportion of phosphatidylserine displayed the opposite trend (Table 4). Supplementation with 18:3n-3 did not result in any significant changes in the proportions of the phospholipid classes compared with the controls, apart from an increase in phosphatidylethanolamine and a decrease in sphingomyelin at 72 weeks (Table 4).

The major polyunsaturated fatty acid components of the phospholipids in spermatozoa from the control birds were 20:4n-6 and 22:4n-6; these phospholipids were almost devoid of n-3 polyunsaturated fatty acids apart from very low concentrations of 22:6n-3 (approximately 2%, w/w) and 22:5n-3 (0.5–1.0%, w/w) (Table 5). Age-related changes in these components included a significant decrease in the proportion of 22:4n-6 and increased concentrations of oleic (18:1n-9) and linoleic (18:2n-6) acids by 72 weeks of age. Dietary supplementation with 18:3n-3 resulted in small but significant effects on these fatty acid profiles. In particular, n-3 supplementation significantly increased the proportion of 22:5n-3 at 39 and 54 weeks, although the maximum amount of 22:5n-3 thus attained was only 5.2% (w/w) of the total phospholipid fatty acids. Most notably, no significant changes

in the amount of 22:6n-3 were induced by 18:3n-3 supplementation, apart from a very small increase (from 1.9% to 2.4%, w/w) at week 54 and a small decrease at week 72. However, the net effect of these small changes in the proportions of individual fatty acids as a result of 18:3n-3 supplementation was to produce considerable decreases in the C_{20–22} n-6:n-3 ratio at 39 and 54 weeks (Table 6). An unforeseen consequence of supplementing the diet with n-3 was a marked increase in the concentration of saturated fatty acids, particularly stearic acid (18:0), in the sperm phospholipid at 72 weeks (Table 5). This resulted in a marked decrease in the polyunsaturated:saturated ratio at this stage (Table 6).

Discussion

The present study has revealed four main findings. First, it is apparent that age-related changes in parameters of semen quality such as sperm concentration and fertilizing ability are accompanied by a range of alterations in sperm lipid composition. Perhaps the most marked change observed in lipid composition with advancing age was the progressive increase in the amount of total lipid per cell. More specifically, the proportion of phosphatidylserine within the total phospholipid

Table 5. The fatty acid compositions of the total phospholipid in spermatozoa from cockerels on a control diet rich in linoleic acid (18:2n-6) and from birds on a diet supplemented with α -linolenic acid (18:3n-3) at 24, 39, 54 and 72 weeks of age

Fatty acid ^a	24 weeks		39 weeks		54 weeks		72 weeks	
	Control	n-3 diet	Control	n-3 diet	Control	n-3 diet	Control	n-3 diet
16:0	13.8 $\pm$ 1.0 ^b	13.9 $\pm$ 1.0 ^b	14.1 $\pm$ 1.0 ^b	13.9 $\pm$ 1.2 ^b	13.9 $\pm$ 1.0 ^b	13.6 $\pm$ 1.2 ^b	15.4 $\pm$ 1.0 ^b	23.1 $\pm$ 1.2 ^{c,e}
18:0	20.0 $\pm$ 1.1 ^b	20.3 $\pm$ 1.1 ^b	19.5 $\pm$ 1.1 ^b	20.2 $\pm$ 1.3 ^b	20.1 $\pm$ 1.1 ^b	20.3 $\pm$ 1.3 ^b	20.4 $\pm$ 1.1 ^b	33.3 $\pm$ 1.3 ^{c,e}
18:1n-9	13.6 $\pm$ 0.6 ^b	13.1 $\pm$ 0.6 ^b	12.2 $\pm$ 0.6 ^b	13.0 $\pm$ 0.7 ^b	14.0 $\pm$ 0.6 ^b	13.6 $\pm$ 0.7 ^b	16.2 $\pm$ 0.6 ^c	16.7 $\pm$ 0.7 ^c
18:2n-6	2.7 $\pm$ 0.2 ^b	2.4 $\pm$ 0.1 ^b	3.4 $\pm$ 0.5 ^b	2.3 $\pm$ 0.1 ^b	3.7 $\pm$ 0.4 ^b	3.7 $\pm$ 0.3 ^b	6.0 $\pm$ 0.8 ^c	2.5 $\pm$ 0.8 ^{b,e}
18:3n-3	0.8 $\pm$ 0.5	1.2 $\pm$ 0.7	nd	nd	nd	nd	nd	nd
20:1n-9	3.2 $\pm$ 0.1 ^b	3.4 $\pm$ 0.1 ^b	3.1 $\pm$ 0.1 ^b	3.4 $\pm$ 0.1 ^b	3.1 $\pm$ 0.1 ^b	3.4 $\pm$ 0.1 ^b	3.0 $\pm$ 0.1 ^b	3.6 $\pm$ 0.2 ^b
20:4n-6	12.5 $\pm$ 0.8 ^b	13.1 $\pm$ 0.8 ^b	11.8 $\pm$ 0.8 ^b	12.1 $\pm$ 0.9 ^b	11.9 $\pm$ 0.8 ^b	12.2 $\pm$ 0.9 ^b	11.0 $\pm$ 0.8 ^b	4.8 $\pm$ 0.9 ^{c,e}
22:4n-6	22.8 $\pm$ 1.2 ^b	23.0 $\pm$ 1.2 ^b	22.9 $\pm$ 1.2 ^b	20.0 $\pm$ 0.5 ^b	21.7 $\pm$ 1.2 ^{b,c}	20.1 $\pm$ 1.5 ^b	17.8 $\pm$ 1.2 ^c	7.5 $\pm$ 1.5 ^{c,e}
22:5n-3	1.0 $\pm$ 0.4 ^b	0.8 $\pm$ 0.4 ^b	1.0 $\pm$ 0.4 ^b	5.2 $\pm$ 0.4 ^{c,e}	0.8 $\pm$ 0.4 ^b	3.4 $\pm$ 0.4 ^{d,e}	0.5 $\pm$ 0.4 ^b	0.6 $\pm$ 0.4 ^b
22:6n-3	2.2 $\pm$ 0.2 ^{b,c}	2.5 $\pm$ 0.2 ^b	2.5 $\pm$ 0.2 ^b	2.4 $\pm$ 0.2 ^b	1.9 $\pm$ 0.2 ^c	2.4 $\pm$ 0.2 ^{b,e}	1.7 $\pm$ 0.1 ^c	1.2 $\pm$ 0.2 ^{c,e}

^aFatty acid compositions are expressed as the percentage (w/w) of the total phospholipid fatty acids; only those fatty acids comprising > 0.5% (w/w) are shown (values are means $\pm$ SD).

^{b,c,d}For comparisons between ages for birds on the same diet, values without a superscript in common are significantly different ($P < 0.05$).

^eDenotes significant difference ($P < 0.05$) between diets for birds of the same age.

nd, not detected.

Table 6. Effects of supplementing the diet of cockerels with α -linolenic acid (18:3n-3) on the ratios of polyunsaturated (P): saturated (S), n-6:n-3 and C₂₀₋₂₂ n-6:n-3 fatty acids in sperm phospholipids at 24, 39, 54 and 72 weeks of age

Ratio	24 weeks		39 weeks		54 weeks		72 weeks	
	Control	n-3 diet	Control	n-3 diet	Control	n-3 diet	Control	n-3 diet
P:S	1.2 $\pm$ 0.1 ^a	1.3 $\pm$ 0.1 ^a	1.2 $\pm$ 0.1 ^a	1.2 $\pm$ 0.1 ^a	1.1 $\pm$ 0.1 ^a	1.2 $\pm$ 0.1 ^a	1.0 $\pm$ 0.1 ^a	0.3 $\pm$ 0.1 ^{b,d}
n-6:n-3	9.5 $\pm$ 1.3 ^a	8.6 $\pm$ 1.6 ^a	10.9 $\pm$ 0.5 ^a	4.5 $\pm$ 0.6 ^{b,d}	13.8 $\pm$ 0.5 ^b	6.2 $\pm$ 0.3 ^{a,d}	15.8 $\pm$ 1.4 ^b	8.2 $\pm$ 3.1 ^a
C ₂₀₋₂₂ n-6:n-3	11.0 $\pm$ 1.5 ^{a,b}	10.9 $\pm$ 1.9 ^a	9.9 $\pm$ 0.4 ^a	4.2 $\pm$ 0.5 ^{b,d}	12.4 $\pm$ 0.5 ^b	5.5 $\pm$ 0.2 ^{c,d}	13.1 $\pm$ 1.1 ^b	6.8 $\pm$ 2.7 ^{a,b,c}

^{a,b,c}For comparisons between ages for birds on the same diet, values without a superscript in common are significantly different ($P < 0.05$).

^dDenotes significant difference ($P < 0.05$) between diets for birds of the same age.

Values are means $\pm$ SD.

was maximal when fertility was high (at 39 weeks) but had decreased significantly by 72 weeks when fertility was declining. The potential importance of long-chain polyunsaturated fatty acids in sperm function was underlined by the decrease in the proportion of 22:4n-6 in the total phospholipid at 72 weeks of age. These changes in lipid composition with age confirm and extend previous observations (Kelso *et al.*, 1996).

The second key result of this study is related to the question of whether the reported predominance of n-6 polyunsaturated fatty acids in avian sperm lipids, in contrast to the predominance of n-3 fatty acids in mammalian sperm lipids, represents a true phylogenetic difference between birds and mammals or is simply an artefact resulting from the feeding of commercial diets to domestic poultry. In general, the fatty acid compositions of the tissue lipids of animals maintained in captivity should be interpreted with some caution since the commercial diets provided may differ greatly in their fatty acid profiles from the natural diets available in the wild. In particular, such commercial diets often contain very high proportions of n-6 fatty acids, to the detriment of n-3 fatty acids (Noble, 1986).

An example of the potential for confusion implicit in data from captive animals is provided by the laboratory guinea-pig, which, in contrast to most mammals, produces spermatozoa characterized by low proportions of 22:6n-3 and high concentrations of 22:5n-6 in the lipids of the brain and retina (Neuringer *et al.*, 1988). Although this discrepancy was originally interpreted as a difference between species, recent work has shown that providing the guinea-pig with a diet enriched in n-3 fatty acids, as opposed to commercial chow, increases the tissue content of 22:6n-3 to typical mammalian concentrations, concomitant with the disappearance of 22:5n-6 (Weisinger *et al.*, 1995). Of greater pertinence to the present study, the low concentrations of 22:6n-3 and correspondingly high concentrations of 22:5n-6 typical of the sperm lipids of the laboratory rat (in contrast to the usual pattern observed for mammalian spermatozoa) were completely reversed by replacing the laboratory chow diet with one supplemented with n-3 fatty acids. Most notably, the resultant increased amount of 22:6n-3 in the rat spermatozoa was accompanied by an improvement in testis maturation and by greater spermatogenesis (Salem *et al.*, 1986).

In the present work, an attempt was made to rectify any dietary deficiency of n-3 fatty acids by supplementing the feed of cockerels with linseed oil, which is rich in 18:3n-3. The aim was to determine whether such n-3 supplementation would result in a transition of the fatty acid profile of spermatozoa of the birds to a pattern more typical of mammals, that is, a switch from n-6 to n-3 fatty acids as the major components of sperm phospholipids. A major conclusion was that the fatty acid profile of the spermatozoa of the cockerels exhibits a distinct resistance to such dietary manipulation. In spite of the provision of an 18:3n-3-enriched diet to the birds for a total of some 49 weeks, little or no increase was observed in the proportion of 22:6n-3, the ultimate endproduct of the chain elongation and desaturation of 18:3n-3, in the sperm phospholipids. The only stage where a significant enhancement in the concentration of 22:6n-3 was observed was at week 54 but this represented only a minor increase, from 1.9% to 2.4% (w/w) of the total phospholipid fatty acids. This effect is trivial in comparison with the amount of this fatty acid routinely observed in mammalian spermatozoa, in which proportions of 22:6n-3 as high as 60–70% (w:w of total phospholipid fatty acids) are achieved in some species, even in the absence of any n-3 supplementation (Salem *et al.*, 1986). A somewhat greater effect of the dietary supplementation with 18:3n-3 was a significant increase in the proportion of 22:5n-3 (the metabolic precursor of 22:6n-3) in the phospholipids at 39 and 54 weeks of age. However, the maximum concentration of 22:5n-3 induced by 18:3n-3 supplementation was only 5.2% (w:w) of the total phospholipid fatty acids. At all ages studied, the major polyunsaturated fatty acids in the sperm phospholipids were 20:4n-6 and 22:4n-6, irrespective of the dietary supplementation. Thus, the sustained feeding of large amounts of 18:3n-3 to the cockerels completely failed to dislodge the ascendancy of n-6 polyunsaturates in the spermatozoa of the birds and certainly did not produce a switch to the typical mammalian pattern. The obvious conclusion from this work is that the preponderance of n-6 polyunsaturated fatty acids reported in the spermatozoa of domestic birds most likely reflects the natural situation and is not an aberration due to the high n-6:n-3 ratios typical of commercial feeds. It would appear, therefore, that the highly distinctive sperm fatty acid compositions of birds and mammals represents a clear-cut biochemical difference between these two classes of higher vertebrate.

Third, the results allow certain inferences to be drawn regarding the metabolism of polyunsaturated fatty acids in relation to spermatogenesis. There is currently a lack of information on the mechanisms involved in the delivery of fatty acyl components from the circulation to the developing spermatozoa and in the selective incorporation of specific fatty acids into the sperm phospholipids. It is clear from the present data that the desaturation and elongation of 18:3n-3 into its C_{20–22} derivatives, or the incorporation of these derivatives into phospholipids, is extremely inefficient in this system. Traditionally it was believed that the synthesis of n-6 and n-3 C₂₂ polyunsaturates from their C₁₈ precursors was performed via the sequential action of $\delta 6$ -, $\delta 5$ - and $\delta 4$ -desaturases plus the intervening elongation steps (Brenner, 1989). However, recent work using a range of mammalian cell types suggests that the direct conversion of 22:4n-6 to 22:5n-6 and of 22:5n-3 to 22:6n-3 via the action of $\delta 4$ -desaturase does not take place.

Instead, it appears that this conversion may occur via a more circuitous route involving microsomal elongation to C₂₄ polyunsaturates followed by retroconversion of these intermediates to 22:5n-6 and 22:6n-3 via peroxisomal β -oxidation (Moore *et al.*, 1995; Sprecher *et al.*, 1995). The present data, which indicate that dietary supplementation with 18:3n-3 results in the appearance of a low concentration of 22:5n-3 in the sperm phospholipid but no notable increase in the proportion of 22:6n-3, suggests that the microsomal elongation of 22:5n-3 and the subsequent peroxisomal retroconversion pathway does not occur in this system. This view is supported by the fact that in cockerel spermatozoa 22:4n-6 appears to be the end product of the desaturation or elongation of 18:2n-6, with no further conversion to 22:5n-6.

An unexpected result of supplementing the diet with 18:3n-3 was the increase it induced in the proportion of saturated fatty acids in the sperm phospholipid at 72 weeks of age, to the detriment of the amount of polyunsaturates of both the n-6 and n-3 series. It would appear that increasing the availability of n-3 fatty acids at this stage somehow inhibits the incorporation of 20:4n-6 and 22:4n-6 into the phospholipid without producing a corresponding increase in the incorporation of 22:5n-3 and 22:6n-3. This deficit in polyunsaturate incorporation is therefore filled by the saturated fatty acids.

The fourth finding of this study was that dietary supplementation with 18:3n-3 produced a significant improvement in semen fertility (from 83% to 97%) at week 39. This corresponded with the increased amount of 22:5n-3 and the decreased n-6:n-3 ratio displayed by the sperm phospholipid at this stage. Thus, although the results of this study indicate that n-6 polyunsaturates probably represent the natural major constituents of avian spermatozoa, the precise balance of n-6:n-3 fatty acids may be crucial to the realization of optimal fertility. It was also noteworthy that the derangement of the polyunsaturate:saturate ratio induced by the 18:3n-3-enriched diet at week 72 was associated with a significant decrease in sperm motility. In summary, the supplementation of the cockerel diet with 18:3n-3 was associated with a significant increase in the fertilizing ability of their spermatozoa at 39 weeks of age and with a corresponding increase in the proportion of n-3 fatty acids.

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